Apical Polarity of Na,K-ATPase in Retinal Pigment Epithelium Is Linked to a Reversal of the Ankyrin-Fodrin Submembrane Cytoskeleton

Doris Gundersen, John Orlowski,* and Enrique Rodriguez-Boulan

Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021; and * Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524

Abstract. In striking contrast to most other transporting epithelia (e.g., urinary or digestive systems), where Na,K-ATPase is expressed basolaterally, the retinal pigment epithelium (RPE) cells display Na,K-ATPase pumps on the apical membrane. We report here studies aimed to identify the mechanisms underlying this polarity "reversal" of the RPE Na,K-ATPase. By immunofluorescence on thin frozen sections, both alpha and beta subunits were localized on the apical surface of both freshly isolated rat RPE monolayers and RPE monolayers grown in culture. The polarity of the RPE cell is not completely reversed, however, since aminopeptidase, an apically located protein in kidney epithelia, was also found on the apical surface of RPE cells. We used subunit- and isoform-specific cDNA probes to determine that RPE Na,K-ATPase has the same isoform (alpha 1) as the one found in kidney. Ankyrin and fodrin, proteins of the basolateral membrane cytoskeleton of kidney epithelial cells known to be associated with the Na,K-ATPase (Nelson, W. J., and R. W. Hammerton. 1989. *J. Cell Biol.* 110:349–357) also displayed a reversed apical localization in RPE and were intimately associated to Na,K-ATPase, as revealed by cross-linking experiments. These results indicate that an entire membrane–cytoskeleton complex is assembled with opposite polarity in RPE cells. We discuss our observations in the context of current knowledge on protein sorting mechanisms in epithelial cells.

THE retinal pigment epithelium (RPE),[1] a single layer of polarized epithelial cells that separates the rods and cones of the retina from the choroid layer, plays an essential role in the development and normal function of the eye (65). Like other transporting epithelia, RPE rests on a basement membrane and is asymmetric in both structure and function. The apical surface contains long specialized microvilli that participate in phagocytosis of dead retinal outer rod segments (17, 32, 64). RPE microvilli have been reported to be motile and to contain myosin 2 and a distinct set of actin binding proteins (42). The apical surface is also involved in the transport of vitamin A during the visual cycle (10).

The basal surface, which rests on a basement (Bruch's) membrane, has numerous infoldings, carries out active transport of ions and metabolites from the underlying choriocapillaris towards the retina, and contains receptors for serum retinol binding protein (3). Retinal attachment depends mainly on the integrity of the RPE layer and appears to depend on both the existence of putative adhesion molecules between RPE and retinal rods and cones and on the net apical to basal transport of fluid by RPE. Although the exact mechanisms responsible for retinal detachment and macular degeneration remain unknown, it has been proposed that the primary changes which lead to these two diseases occur in the RPE (19).

A curious feature of RPE is the apical localization of Na,K-ATPase pumps (3, 4, 51), in contrast to other transporting epithelia, where these pumps are basolateral (21). Na,K-ATPase activity is essential for the maintenance of Na and K electrochemical gradients across the plasma membrane in all cells and for a variety of transepithelial ion transport processes (18).

Na,K-ATPase is a heterodimeric integral membrane protein formed by a catalytic subunit, alpha, with the binding sites for ATP and cardiac glycosides, and a glycosylated beta subunit. Although no function has yet been assigned to the beta subunit, it is believed to participate in the regulation of the function and membrane insertion of the alpha subunit (25). Three different isoforms of the alpha subunit have been isolated from brain (11, 48–50, 53), alpha 1, alpha 2 and alpha 3. It is unclear if the opposite localization of Na,K-ATPase in kidney and RPE may be accounted for by the expression of different, tissue-specific isoforms.

Na,K-ATPase, ankyrin, and fodrin have been shown to be colocalized along the basolateral margins of confluent MDCK cells, intact kidney proximal tubules (20, 35), and isolated intestinal epithelium (1). In MDCK cells, all of these proteins form a macromolecular complex via interaction of

1. *Abbreviations used in this paper*: DSP, dithiobis(succimidylpropionate); RPE, retinal pigment epithelium.

the alpha subunit with ankyrin and of ankyrin with fodrin (30, 34, 36). This membrane complex is relatively detergent soluble and unpolarized in subconfluent MDCK cells but polymerizes into insoluble aggregates upon confluency (33); the basolateral localization of these aggregates is apparently induced by intercellular adhesion via L-CAM (27, 36). Development of this polarized, contact-induced, membrane-cytoskeletal network is believed to play a key role in the morphogenesis and maintenance of the polarized epithelial phenotype (45). Observations in the dystrophic rat RPE (7) suggest that cell-cell contacts may also play a role in the maintenance of RPE polarity: as the pathological process induces a breakdown of the junctional complexes, the Na,K-ATPase activity becomes redistributed from the apical surface to the lateral and basal regions of the RPE cells.

Here, we localize by immunocytochemistry both subunits of Na,K-ATPase and report that the alpha isoform expressed in RPE is identical to the kidney one. We find that the reversed polarity of Na,K-ATPase is accompanied by a reversed (apical) distribution of ankyrin and fodrin, which interact closely with Na,K-ATPase in RPE, as previously described in MDCK cells (31, 36, 37).

Materials and Methods

Preparation of Antibodies

Na,K-ATPase was prepared from freshly isolated rat kidney outer medulla according to Jorgensen (18). Alpha and beta subunits were excised from a polyacrylamide gel and, after suspension in complete Freund's adjuvant, were injected intradermally into New Zealand white rabbits; the rabbits were boosted with four intradermal injections in incomplete Freund's adjuvant. Immobilized alpha and beta subunits were used to prepare affinity-purified antibodies. Briefly, purified alpha and beta subunits were separated by denaturing polyacrylamide gel (10%) and blotted onto diazotized paper (43). Paper strips containing only alpha or beta subunits were cut, incubated with the appropriate antiserum, and washed to remove non-specifically bound antibody. Specific antibodies were eluted in a minimum volume of a low pH buffer and quickly neutralized. The specificity of the antibodies was determined by Western blot analysis against purified rat kidney alpha and beta subunits. Fodrin and ankyrin antibodies (36) were kindly provided by Dr. James Nelson (Institute for Cancer Research, Philadelphia, PA).

Cell Isolation and Culture

The procedure used to generate primary RPE cultures is essentially as described by Mayerson et al. (24a). Eyes are enucleated from 7-d-old Long Evans rats, rinsed two to three times with a balanced salt solution (BSS), incubated 40 min in an enzyme solution containing 105 U/ml collagenase, 50 U/ml testicular hyaluronidase, pH 7.0, followed by 50 min in 0.1% trypsin in BSS. The eyes are then opened by a circumferential incision below the ora serrata, the retina is lifted off, and the single layer of RPE cells is peeled from both the retina and the choroid. After rinsing and a gentle trypsinization (0.1%) the cells are plated in DMEM containing 10% fetal calf serum. The following day the medium is changed to DMEM containing 2% fetal calf serum. Rat RPE cells in culture could not be serially passaged.

Semithin Frozen Sections and Transmission Electron Microscopy

0.5 μm frozen sections were processed for immunofluorescence as described (58). Samples were photographed with an epifluorescence microscope (Leitz). Cells grown on filters for 2 wk were fixed in 2% glutaraldehyde treated with ruthenium red and processed for transmission electron microscopy (44). Sections were either stained with uranyl acetate and lead citrate or examined unstained in a JEOL 100 CX electron microscope.

Immunoprecipitation

Confluent monolayers on filters were rinsed four times in DMEM without methionine/cysteine, then labeled with the same medium containing 1 mCi Trans [^{35}S]label (ICN Biomedicals, Costa Mesa, CA) per milliliter (1 Ci = 37 GBq) for 5 h. The filters were rinsed five times in PBS, the cells were solubilized, and fodrin and ankyrin were immunoprecipitated (23). Na,K-ATPase was immunoprecipitated according to described methods (33). Metabolically labeled samples were subjected to SDS-PAGE (22) under reducing conditions. After fixation (40% methanol/10% acetic acid for 30–60 min) gels were rehydrated in distilled water, then impregnated with sodium salicylate (1 M solution for 30 min). Dried gels were autoradiographed on Kodak XAR-5 film at −70°C for varying lengths of time.

Isolation of RNA

RPE cultures were rinsed with ice-cold buffered saline and then lysed in 4 M guanidinium thiocyanate at 2×10^7 cells per ml (13). The extract was passed through a 23-gauge needle five times to reduce viscosity and then centrifuged at 35,000 rpm through a cushion of 5.7 M CsCl for 12 h. The RNA pellet was rinsed with ethanol and resuspended at 1 mg/ml in distilled water.

Northern Blot Analysis

Northern blots were prepared as described previously (40). Subunit-specific DNA restriction endonuclease fragments (~300 bp) were isolated (63) from rat Na-K,ATPase alpha 1 (Nar I–Stu I, nt 89–331), alpha 2 (Xho II–Xho II, nt 4,285–5,026), alpha 3 (Pst I–Sma I, nt 51–331), and beta (Nco I–Stu I, nt 459–759) subunit cDNAs. These DNA fragments were radiolabeled with (alpha ^{32}P)dCTP by using an oligolabeling kit (Pharmacia LKB Biotechnology Inc.) developed from the random primer method of Feinberg and Vogelstein (12). The radiolabeled DNA fragments ($\sim 1 \times 10^9$ cpm/μg) were added to the hybridization solution (2×10^6 cpm/ml) and used as probes for Northern blots.

Immunoblotting

The method of Towbin et al. (59) was used to detect electrophoretically transferred proteins on nitrocellulose strips. The immunodetection was performed using alkaline phosphatase–conjugated second antibodies (Promega Biotec, Madison, WI). The chicken alpha subunit monoclonal antibody (which cross-reacts with the rat enzyme) used in some Western blots was provided by Dr. D. Fambrough (Carnegie Institution of Washington, Baltimore, MD).

Cross-Linking

Confluent monolayers were labeled with Trans [^{35}S]label overnight, rinsed five times with PBS, cross-linked with dithiobis(succinimidylpropionate) (DSP, 1 mg/ml in PBS) (Pierce Chemical Co., Rockford, IL) two times for 30 min at 37°C, quenched with DMEM for 10 min, rinsed five times with PBS, and then immunoprecipitated as described above.

Results

Polarity of RPE Cells

Since RPE cells constitute a polarized epithelium in vivo it was important to establish that they maintain these characteristics when grown in vitro. Fig. 1 *A* shows their morphology in vivo. They are typically polygonal and are sometimes binucleated. The apical microvilli are not readily visible due to interdigitation with the rods and cones of the outer segment of the retina. RPE cells rest on a basement membrane, Bruch's membrane, and their lateral borders are distinct.

Rat RPE cells grown in culture retain some of their epithelial morphological characteristics. Pigment granules and premelanosomes are characteristic, radiating from a central densely populated cytoplasmic region, but decrease in number as the cells divide. The appearance of the cells in the central areas of the culture (Fig. 1 *B*), hexagonal and tightly

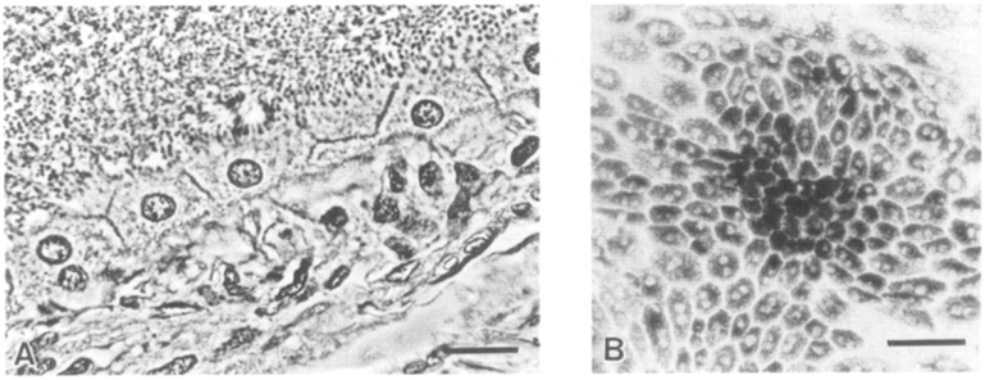


Figure 1. Retinal pigment epithelium cells in situ and in vitro. (*A*) Paraffin section of intact eye, note distinct intercellular limits of RPE cells. (*B*) Primary culture of RPE monolayer grown on plastic for 10 days; note hexagonal morphology and pigment granules. Bars: 10 μm (*A*); 50 μm, (*B*).

packed, most closely resemble RPE cells in vivo. It was not possible to measure transmonolayer electrical resistance to assess the integrity of the tight junctions because rat RPE cells in culture did not form continuous monolayers on nitrocellulose or polycarbonate filters. However, immunocytochemistry clearly demonstrated the presence of tight junctions.

Cingulin and ZO-1, two proteins peripherally associated with the cytoplasmic aspect of epithelial tight junctions (9, 52), exhibited in cultured RPE cells a similar distribution to that observed in polarized epithelial cell lines, such as MDCK (9, 52). Z0-1 distinctly localized into areas of cell–cell contact, forming a typical fluorescent ring around the top of the cell (Fig. 2 *B*), seen by focusing up and down the monolayer. Cingulin displayed a similar pattern (data not shown). Free cell borders at the periphery of subconfluent cell colonies were not stained by antibodies to either of these proteins. The presence of the characteristic ZO-1 and cingulin rings indicates that well-developed tight junctions are found in cultured RPE cells, suggesting that these monolayers maintain the polarity properties of the native epithelium. Tight junctions were also demonstrated by ruthenium red staining; when added from above, the dye did not penetrate across the monolayer and usually stopped at the top of the intercellular space (data not shown).

Electrophoretic Analysis and Immunofluorescence Localization of Na,K-ATPase

Ouabain binding assays and cytochemical procedures have shown that Na,K-ATPase is localized on the apical surface of RPE (16, 26, 28); this enzyme, however, is located on the basolateral surface of most other transporting epithelia, such as kidney, intestine, etc. (21) as well as in kidney epithelial cell lines, like MDCK. The apical localization of RPE Na,K-ATPase provides a natural "mutant" to study the mechanisms that regulate its polarized localization in epithelial cells.

Na,K-ATPase is composed of two subunits, alpha, the catalytic subunit, and beta, of unknown function. With the goal of immunolocalizing the individual subunits, we raised and affinity purified polyclonal antibodies against each of the subunits (see Materials and Methods). The antibodies reacted specifically (by Western blot analysis) with either the alpha or the beta subunits of purified rat kidney Na,K-ATPase (Fig. 3 *A*).

The alpha and beta subunits of RPE Na,K-ATPase migrated with an apparent electrophoretic mobility of 95 kD, identical to the mobility of the alpha and beta subunits of MDCK cells (Fig. 3 *B*), notwithstanding the different tissue (RPE vs. kidney) and species (rat vs. dog) origins. There seemed to be no indication of multiple alpha subunit bands, commonly found in brain membrane preparations, indicating the existence of distinct alpha isoforms. By immunoprecipitation with alpha subunit antibody of samples crosslinked with the bifunctional (thiol cleavable) reagent dithiobis(succinimidylpropionate) (DSP), a band with the typical mobility of the beta subunit was detected in MDCK and RPE. This band had identical mobility in both tissues (Fig. 3 *C*); its lower level in MDCK cells may be attributed to lower accessibility of the cross-linker to the basolateral surface of MDCK cells than to the apical surface of RPE. After treatment with endoglycosidase F to examine the mass of the core protein, the beta subunits of both MDCK and RPE changed

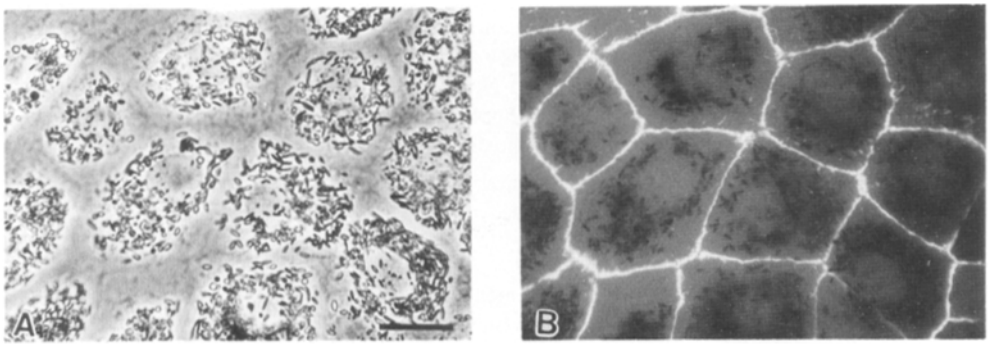


Figure 2. Expression of ZO-1 in RPE monolayers. Phase (*A*) and immunofluorescence (*B*) micrograph of primary RPE monolayers stained with ZO-1 antibody. Note the existence of continuous tight junctions. Bar, 5 μm.

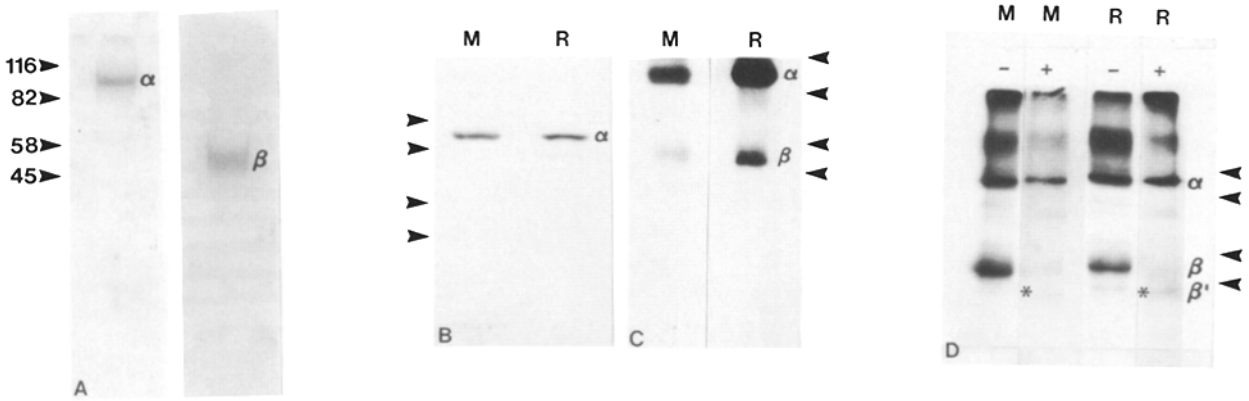


Figure 3. Comparison of RPE and MDCK Na,K-ATPases. (*A*) Affinity-purified antibodies raised to both the alpha and beta subunit of rat kidney Na,K-ATPase, blotted against purified rat kidney enzyme, showing the specificity of both subunit antibodies. (*B*) Immunoprecipitation of basolateral Na,K-ATPase alpha subunit from MDCK (*M*) monolayers, and apical alpha subunit from RPE (*R*) from monolayers labeled with Trans [^{35}S]label added overnight. Note the similar electrophoretic mobilities in both tissues. (*C*) Immunoprecipitation of metabolically labeled MDCK (*M*) and RPE (*R*) monolayers cross-linked with DSP with an alpha subunit specific antibody. Monolayers were grown on polycarbonate filters; DSP was added from the apical (RPE) or from the basolateral (MDCK) sides. PAGE was performed under reducing conditions. Note similar mobilities of the beta subunits in both tissues. (*D*) Migration of alpha and beta subunits in SDS-PAGE, labeled, and cross-linked as described in *C*, either undigested (−), or digested (+), with endo F in MDCK (*M*) and RPE (*R*) monolayers. Both glycosylated beta subunits shift to a lower molecular mass indicated by asterisk (~35 kD) in MDCK and RPE cells, while the unglycosylated alpha subunit remains unaffected.

in mobility to two bands with apparent M_r of 35 and 38 kD, identical in both tissues (Fig. 3 *D*). The smaller 35-kD band is characteristic of the nonglycosylated beta 1 subunit; the larger band may comprise a partially endo F resistant species, as previously reported (38).

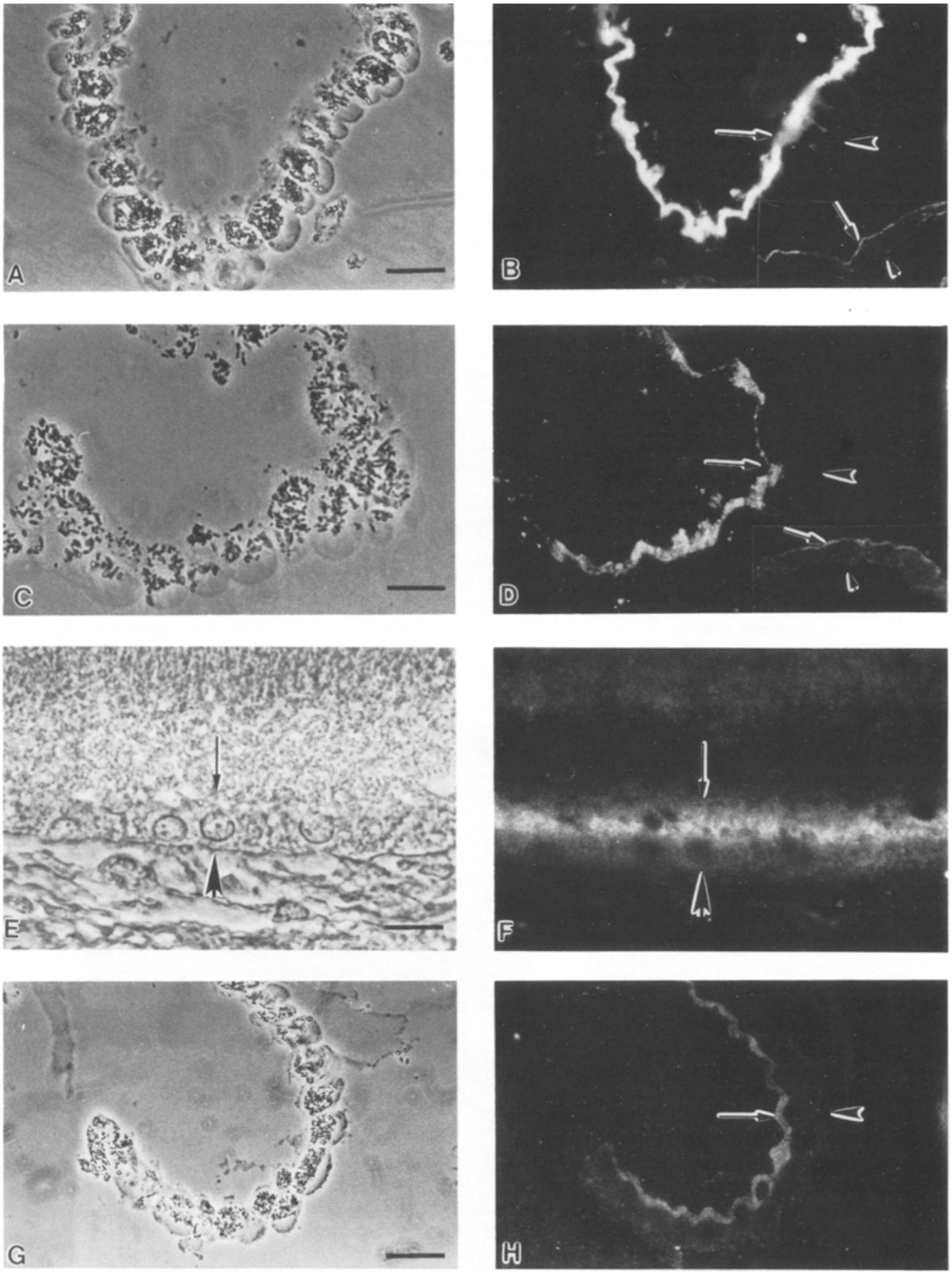
We used the polyclonal antibodies for immunofluorescence on 1-μm frozen sections. Control experiments showed that both subunits were localized on the basolateral surface of kidney tubular cells and MDCK cells (data not shown). In RPE fixed immediately after isolation, the alpha and beta subunits were found to be restricted to the apical surface (Fig. 4, *A–D*). RPE cells grown in culture also displayed alpha and beta subunit Na,K-ATPase staining on the apical surface (Fig. 4, *insets*); the decreased intensity of staining may be attributed to the decreased number of microvilli and the flatter more extended morphology of the cultured cells. The localization of Na,K-ATPase was also studied in a paraffin section of the whole eye (Fig. 4, *E* and *F*) to exclude possible artifacts of cell manipulation. After staining with a beta subunit antibody, the long apical microvilli surrounding the rods and cones of the retina appeared stained, whereas the basolateral surfaces were devoid of staining. A possible contribution of photoreceptor beta subunit to the microvillar staining observed in Fig. 4 *F* cannot be completely excluded but a major contribution is unlikely since we observe a similar apical distribution of beta subunits in isolated RPE (Fig. 4 *D*). These experiments clearly showed that both subunits of Na,K-ATPase are apical in RPE cells.

To determine whether other integral plasma membrane markers are also reversed, we studied the localization of aminopeptidase N. Like kidney, intestinal and other transporting epithelia, RPE displayed APN on the apical surface (Fig. 4, *G* and *H*). Other cellular markers (e.g., gamma-glutamyltransferase, integrin receptors) have been shown to be present with similar distributions in RPE and other transporting epithelia (41, 57). Furthermore, influenza virus budding occurs from the apical surface, whereas VSV budding occurs from the basolateral surface of RPE (O'Day, W., E. Rodriguez-Boulan, and D. Bok, manuscript in preparation; Gundersen, D., and E. Rodriguez-Boulan, unpublished results), as previously described in MDCK cells (46). Therefore, it appears that polarity reversal is restricted to Na,K-ATPase (and perhaps to a reduced set of other proteins) in RPE.

RPE Expresses the Alpha 1 Kidney Isoform

The alpha subunit exists as three distinct isoforms (53). Kidney cells express the alpha 1 isoform while the alpha 2 and alpha 3 isoforms are expressed predominantly in the brain (53). This tissue-specific expression of the alpha subunit isoforms is developmentally regulated; however, the functional significance of this diversity has yet to be determined. Since RPE cells are derived from the neural ectoderm it was conceivable that these cells might contain either one of the neural specific isoforms and that this could account for the reversal in polarity of the Na,K-ATPase. As shown in Fig. 5, comparative Northern blot analysis of rat brain (which expresses all three isoforms) and RPE mRNAs with alpha isoform-specific cDNA probes indicated that RPE cells express

Figure 4. Apical expression of Na,K-ATPase subunits and aminopeptidase in RPE monolayers. (*A–D*) Apical localization of both alpha (*A* and *B*), and beta (*C* and *D*) subunits, with affinity-purified polyclonal antibodies to Na,K-ATPase in 1.0 μm frozen sections of freshly isolated sheets of RPE monolayers, and on primary cultures (*insets*) grown on 10% gelatin. The apical surface can be determined by the



presence of prominent pigment granules in the phase-contrast micrograph (*A* and *C*). Arrows indicate stained apical membrane, arrowheads point to unstained basal surface. (*E* and *F*) Paraffin section of whole eye stained with beta subunit Na,K-ATPase antibody. Immunofluorescent staining is present on the apical microvilli (*arrows*) while the basal surface is negative (*arrowheads*). (*G* and *H*) Immunofluorescence localization of aminopeptidase N, a known apical marker in other polarized epithelia, also present on the apical (*arrows*) surface of RPE monolayers. *A*, *C*, *E*, and *G* are phase-contrast micrographs. *B*, *D*, *F*, and *H* represent the corresponding fluorescence pictures. Bars: 10 μm (*A–F*); 20 μm (*G* and *H*).

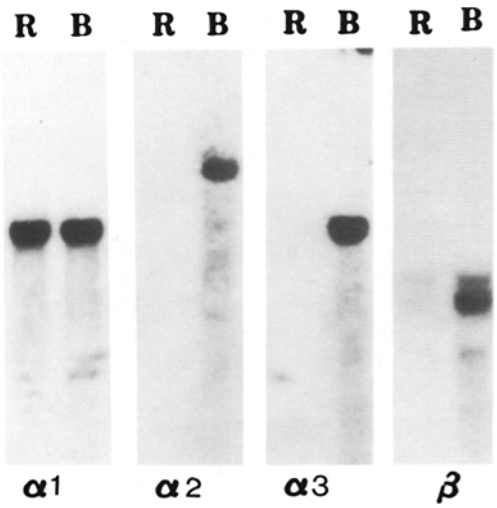


Figure 5. Analysis of mRNA in RPE vs. brain. Northern blot analysis of mRNA isolated from RPE (*R*) and rat brain (*B*), probed with the three known alpha-specific cDNA probes, originally isolated from brain, and a beta-specific probe for Na,K-ATPase. The alpha 1 isoform is the same one present in kidney. The four different beta subunit mRNAs are present in much lower amounts than brain, however, their sizes are similar.

the alpha 1 isoform, which is the only isoform present in kidney.

In most rat tissues, beta 1 subunit–specific cDNAs hybridize to four distinct mRNA species. The abundance of each particular species varies considerably in different tissues. In kidney, brain, and heart equivalent amounts of alpha and beta mRNA are expressed suggesting that they may be coordinately regulated; however, in other tissues, such as muscle, stomach, and RPE the amount of beta mRNA synthesized is much less than the alpha mRNA detected (63). These four different beta mRNA species have been found to be identical at the protein level differing only at their 5′ or 3′ untranslated regions. These differences arise from multiple initiation and polyadenylation sites and there is evidence that these RNAs are transcribed from a single gene (62).

RPE was found to express similar size beta subunit transcripts as in brain (Fig. 5) although at considerably lower amounts. The low levels of expression of beta subunit mRNA in RPE cells is compatible with the possible expression of alternative beta isoforms, since very recently it was shown that a second isoform (denominated beta 2) exists in some (but not all) tissues with low expression of beta 1 (24, 50) (see Discussion).

These results showed that the opposite localization of RPE and kidney Na,K-ATPase cannot be accounted for by expression of a different alpha isoform.

Reversal of the Ankyrin-Fodrin Submembrane Cytoskeleton in RPE Cells

Since the analysis of the alpha and beta subunits of the Na,K-ATPase did not reveal any obvious differences at either the protein or the mRNA level, the reversed polarity of this enzyme may reflect a partial or full reversal in the polarity of the RPE cell itself. Since the polarization of Na,K-ATPase has been related to the establishment of a basolateral anky-

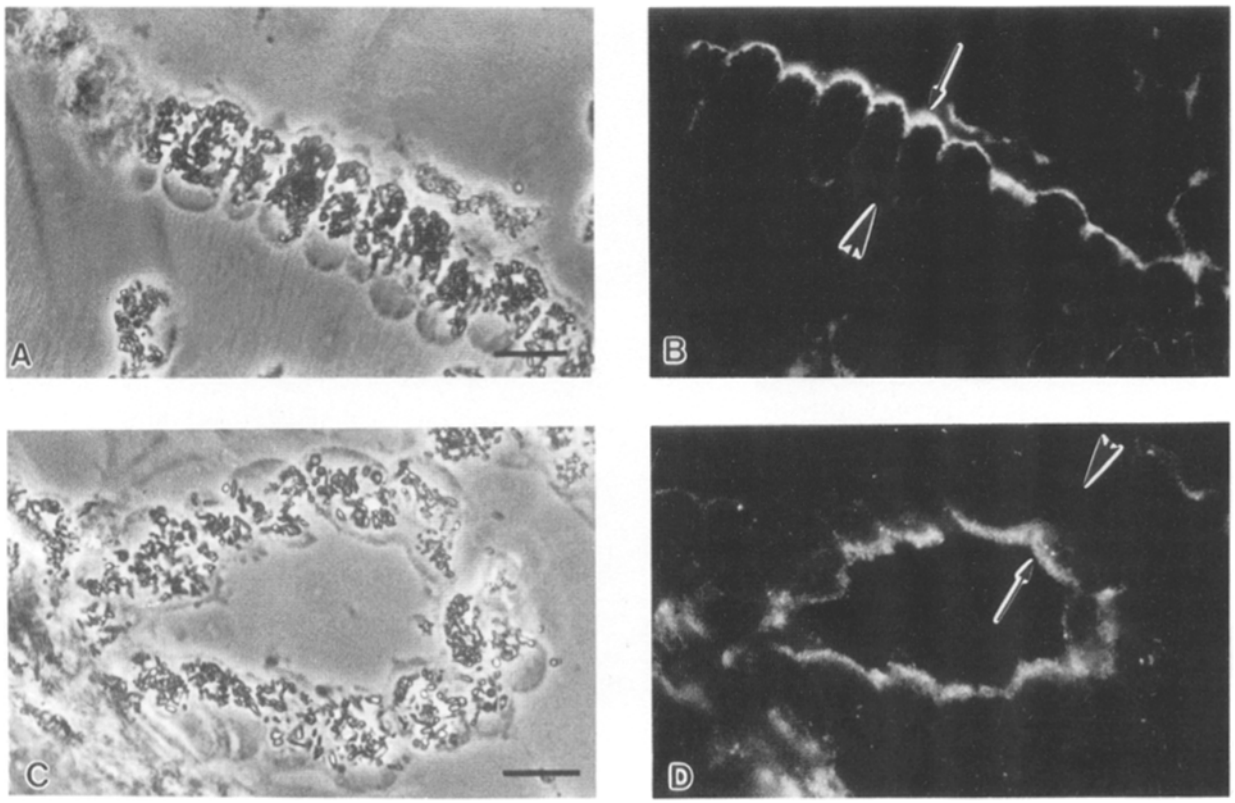


Figure 6. Immunofluorescence localization of fodrin and ankyrin. Apical localization of fodrin (*A* and *B*) and ankyrin (*C* and *D*), in 1.0-μm frozen sections of RPE sheets from freshly enucleated eyes. The apical surface can be recognized by the presence of pigment granules immediately underneath. Bar, 10 μm.

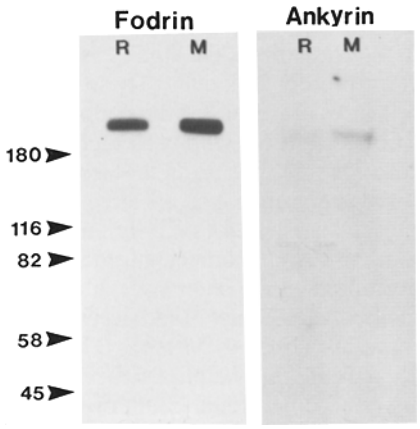


Figure 7. Mobilities of fodrin and ankyrin. Immunoprecipitation of fodrin and ankyrin from RPE (*R*) and MDCK (*M*) cells. Both antigens have identical mobilities on SDS-PAGE in the two different cell types.

rin/fodrin cytoskeleton in MDCK cells (33–35), we examined the localization of these cytoskeletal proteins in RPE cells. Localization of ankyrin and fodrin by immunofluorescence showed that they were both restricted to the apical surface, like Na,K-ATPase (Fig. 6). Immunoprecipitation of ankyrin and fodrin revealed bands of 220 and 240 kD, respectively (Fig. 7), in both MDCK and RPE cells. They were recognized by the same antibody and displayed identical electrophoretic mobilities in both cell types, notwithstanding their different tissue and species origin.

Nelson and Veshnock (35) have described a high affinity interaction between Na,K-ATPase and ankyrin, which is linked to fodrin (the nonerythroid form of spectrin), as shown in the red blood cell (2). We decided to study whether such a complex also occurs along the apical surface of RPE cells. Monolayers of RPE prelabeled with Trans [^{35}S]label were cross-linked with DSP, immunoprecipitated with ankyrin or fodrin specific antibodies, and the immunoprecipitates analyzed by SDS-PAGE (under reducing conditions, to reverse the cross-linking). Parallel unlabeled samples were analyzed by blotting with an antibody against the alpha subunit of Na,K-ATPase. Cross-linked samples immunoprecipitated with ankyrin antibody contained a major band at 95 kD (Fig. 8 *A*, *left*), which was not present in noncross-linked samples (Fig. 7). Immunoblot analysis with Na,K-ATPase alpha subunit antibody of a parallel sample revealed a single band running at ~95 kD (Fig. 8 *B*, *left*), suggesting that the alpha subunit had been cross-linked to ankyrin. Similar results were observed for fodrin, i.e., a band with the mobility and antigenicity of the alpha subunit was detected after cross-linking. However, the levels of alpha subunit cross-linked to fodrin were much lower, since much longer incubations with substrate were needed for its detection by enzymatic immunoblot (overnight vs. 10 min for ankyrin samples) and by immunoprecipitation (only a faint

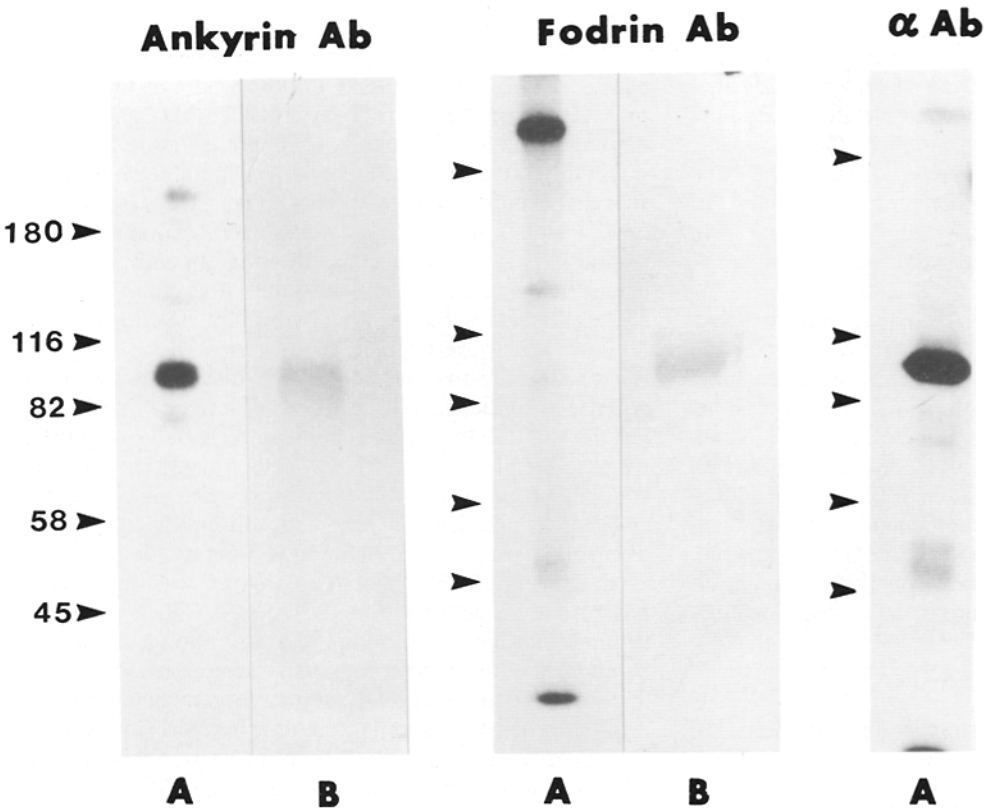


Figure 8. Cross-linking between Na,K-ATPase and the ankyrin-fodrin cytoskeleton. Cultured RPE monolayers were labeled overnight with Trans [^{35}S]label, cross-linked with DSP, and immunoprecipitated with ankyrin, fodrin, and an alpha subunit-specific antibody (control). Parallel unlabeled samples were immunoblotted with an alpha subunit-specific antibody and detected by an enzymatic reaction (see Materials and Methods). Immunoprecipitates and samples destined for immunoblot were run on parallel, identical gels. Different shapes of immunoprecipitated and blotted bands may be attributed to the use of a different comb for the latter, to accommodate a larger volume. (*A*) Metabolically labeled samples. Note bands with the mobility of ankyrin (220 kD), fodrin (240 kD), and alpha subunit (95 kD). (The latter is very faint in the fodrin sample). (*B*) Immunoblots. Note the specific staining of a band with the mobility of the alpha subunit (95 kD) in the samples immunoprecipitated with fodrin and ankyrin antibodies (*B*), suggesting the existence of a complex between Na,K-ATPase, ankyrin and fodrin. Note, however, that a much longer incubation with the substrate was required to observe the 95-kD band in the fodrin (overnight incubation) than in the ankyrin (~10-min incubation) immunoprecipitate. Accordingly, a much stronger 95-kD band was observed in the immunoprecipitate (compare the ankyrin and fodrin A samples).

band was observed at 95 kD). This is consistent with the observation that ankyrin is directly linked to Na,K-ATPase in a molar ratio of 1:1 (35), likely via the alpha subunit (31), whereas no direct interaction exists between fodrin and Na,K-ATPase.

The close proximity between Na,K-ATPase, ankyrin, and fodrin detected by the cross-linking experiments suggests that these proteins form a complex along the apical surface of RPE cells similar to the one detected on the basolateral surface of MDCK cells.

Discussion

The results of this report provide several important observations on the molecular basis of the "reversed polarity" of Na,K-ATPase in RPE. First, we have demonstrated by immunofluorescence that both alpha and beta subunits are expressed on the apical surface of RPE cells, i.e., with a polarity opposite to that seen in kidney cells. (Previous work had only demonstrated Na,K-ATPase "activity" on the apical surface.) Second, at the protein and mRNA levels, the kidney and RPE alpha subunits appear to be identical. In spite of the neural ectodermal origin of RPE, only the alpha 1 subunit (from the three subunits expressed in brain) is detected in this epithelium. Third, low levels of beta transcripts were detected, which opens the possibility that a different beta subunit may be expressed in RPE. Fourth, the reversal of Na,K-ATPase is accompanied by a similar reversal of the ankyrin-fodrin submembrane cytoskeleton, which remains intimately associated with the enzyme in the apical surface.

The observation that both subunits are expressed on the apical surface of RPE is consistent with available knowledge about the biology of the enzyme. Current evidence indicates that alpha and beta subunits are inserted independently into the endoplasmic reticulum, which is where they associate stoichiometrically quickly after glycosylation of the beta subunit (56). This stoichiometric complex is necessary for functional Na,K-ATPase activity. When beta subunit glycosylation is inhibited by tunicamycin alpha-beta complexes will form, although much less efficiently (14). Na,K-ATPase exit from the endoplasmic reticulum and transport to the cell surface are regulated by the beta subunit (15, 25, 55); however, the beta subunit may be capable of reaching the plasma membrane without interacting with the alpha subunit (Mirchoff, A. K., J. W. Bowen, S. C. Yiu, and A. A. McDonough. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:A874[Abstr.]). Delivery to the cell surface occurs with a lag time of 45–50 min, which is in agreement with the transit time for other membrane proteins (5, 56). The functional and biogenetic interdependence between both subunits of Na,K-ATPase (not observed in other cation-translocating ATPases) suggests that sorting information to reach the appropriate epithelial surface domain needs only be contained in one of the two subunits.

This tissue specific expression of the alpha subunit isoforms is developmentally regulated, however, the functional significance of this diversity has yet to be determined (53). Since RPE cells are derived from the neural ectoderm, we considered the possibility that these cells might contain either one of the neural specific isoforms and that this could account for the reversal in polarity of the Na,K-ATPase. As shown in Fig. 6, comparative Northern blot analysis of rat brain and RPE mRNAs with alpha isoform-specific cDNA probes indicates that RPE cells express the alpha 1 isoform, which is the only isoform present in kidney. These results confirm and extend (in that we searched for three alpha isoforms) results by McGrail et al. (26), who used alpha- and alpha+- (now called alpha 1 and 2) specific antibodies before the cloned cDNA probes were available to all three known alpha isoforms. Their results suggested that only the alpha subunit (alpha 1 according to current nomenclature) was expressed in RPE. Photoreceptors of the outer layer of the retina, which directly opposes the RPE layer (and derive from the same optic cup primordium), express the alpha 3 isoform (47). Furthermore, although the three alpha isoforms have been detected in the bilayered ciliary epithelium of the bovine eye, the pigmented epithelial layer of this epithelium, which is directly continuous with the RPE cells (and derives, like RPE, from the outer region of the optic cup), only expresses the alpha 1 isoform, yet the enzyme is distributed along the basolateral surface (16). Thus, the alpha isoform type does not seem to be involved in determining the apical or basolateral localization of the enzyme in different epithelia.

Our results do not discard a possible role of the beta subunit in determining the localization of Na,K-ATPase. Northern blot analysis of RPE showed that the mRNAs encoding the beta subunit are the same sizes as in brain and kidney, but are expressed at much lower levels. Results reported during the last year have demonstrated that the beta subunit consists of at least two different isoforms, beta 1 and beta 2 (24, 50). Since the probe available to us was beta 1 and, since several tissues which express low levels of beta 1 (like pineal gland and thymus) have been shown to express beta 2, it is possible that RPE will eventually be found to express an alternative form of beta subunit. Although this alternative subunit may be beta 2, it is perhaps more likely that it will turn out to be a different beta subunit, since many tissues that express low beta 1 levels do not express (or express low levels of) beta 2 (e.g., liver, spleen, mammary gland, or lung). This opens the possibility that an entire family of beta subunits may exist, and that alternative combinations of alpha and beta subunits would result in functional Na,K-ATPases with different cellular locations.

Two mechanisms that control the distribution of Na,K-ATPase in epithelial cells have been described. Caplan et al. (6) showed that the alpha subunit is vectorially targeted to the basolateral surface of MDCK cells, suggesting that intracellular sorting into basally targeted vesicles may be responsible for its polarized surface distribution (45, 61). A complementary mechanism, believed to operate mainly during establishment of the epithelial monolayer, was suggested by Nelson and his collaborators (36). Na,K-ATPase was shown to bind with high affinity and in a 1:1 ratio to the ankyrin component of an ankyrin-fodrin submembrane cytoskeleton that localizes to the basolateral membrane when the monolayer becomes confluent (31, 33–35). L-CAM (uvomorulin, E-cadherin) was also shown to form part of this complex (37). An important recent observation is that L-CAM appears to induce the polarized distribution of the Na,K-ATPase when expressed in fibroblasts by transfection of its cDNA, suggesting that a similar role may be played in epithelial cells (36).

We clearly show here that, like Na,K-ATPase, ankyrin and

fodrin also display a striking reversal of their surface expression in RPE (as compared with kidney cells and intestinal cells). Amerongen et al. (1) reported that, in isolated intestinal epithelium, basal (but not lateral) enzyme, ankyrin, and fodrin are endocytosed, whereas the same proteins are more stable on both lateral and apical membranes. It is unlikely that the apical distribution of these proteins we observe in RPE be a consequence of endocytic removal of (putative) basolateral pools of the proteins, since we observe similar distribution in situ of ATPase (antibodies against ankyrin and fodrin did not work in paraffin sections) and we see no traces of lateral staining for these proteins.

With the available evidence, it is not possible to establish whether the apical expression of ankyrin and fodrin is the cause or the consequence of the reversal of Na,K-ATPase distribution. According to Nelson's hypothesis, a decreased expression of L-CAM (or the expression of a molecule with equivalent properties on the apical surface) might lead to reversed apical polarity of the ankyrin-fodrin cytoskeleton and consequent reversal of the Na,K-ATPase. In fact, our own unpublished results (Gundersen, D., and E. Rodriguez-Boulan, manuscript in preparation) indicate that L-CAM is not expressed in RPE. Grunwald (8) has proposed that a different cadherin is expressed in RPE; perhaps this molecule lacks the ability to induce the formation of the ankyrin-fodrin submembrane cytoskeleton.

The specialized function of RPE in phagocytosing dead outer rod segments may require the presence of other unique adhesion molecules on their apical surfaces. Sweadner and co-workers (Sweadner, K., H. Antonicak, and M. Schachner. 1989. *Soc. Neuro. Abstr.* 15:568) recently reported that the beta 2 subunit exhibits virtual identity with AMOG (glial cell adhesion molecule). Perhaps this beta 2 subunit, or another beta subunit, can have a dual function in providing some type of adhesion on the apical surface between the RPE layer and the rods and cones of the retina, while also directing the Na,K-ATPase, ankyrin, fodrin complex to the apical surface of RPE cells. We are currently exploring whether lack of L-CAM expression or expression of alternative adhesion molecules on either the apical or the basolateral surface are main determinants of the reversed polarity of RPE.

We thank Dr. Andre Le Bivic for help and discussion during the course of this work; Dr. Paula Traktman for her advice with the preparation of mRNA; Mrs. Susan Pettersen and Ms. Michelle Garcia for excellent technical assistance; and Ms. Polly Lai and Ms. Lori van Houten for photographic work.

This work was supported by grants from the National Institutes of Health (NIH) (GM 34107 and EY 07201) and by a grant in aid from the New York Heart Association to E. Rodriguez-Boulan. John Orlowski was supported by a postdoctoral fellowship from the Medical Research Council of Canada and by NIH grant HL 28573 to Dr. Jerry B. Lingrel, University of Cincinnati College of Medicine.

Received for publication 28 September 1990 and in revised form 15 November 1990.

References

1. Amerongen, H. M., J. A. Mack, J. M. Wilson, and M. R. Neutra. 1989. Membrane domains of intestinal epithelial cells: distribution of Na,K ATPase and the membrane skeleton in adult rat intestine during fetal development and after epithelial isolation. *J. Cell Biol.* 109:2129–2138.
2. Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273–304.
3. Bok, D. 1982. Autoradiographic studies on the polarity of plasma membrane receptors in RPE cells. *In* The Structure of the Eye. H. Field, editor. Elsevier North Holland, New York. 247–256.
4. Caldwell, R. B., and B. J. McLaughlin. 1984. Redistribution of Na-K-ATPase in the dystrophic rat retinal pigment epithelium. *J. Neurocytol.* 13:895–910.
5. Caplan, M., B. Forbush, G. Palade, and J. Jamison. 1990. Biosynthesis of the Na,K-ATPase in MDCK cells. *J. Biol. Chem.* 6:3528–3534.
6. Caplan, M. J., G. E. Palade, and J. D. Jamieson. 1986. Newly synthesized Na,K-ATPase alpha-subunit has no cytosolic intermediate in MDCK cells. *J. Biol. Chem.* 261:2860–2865.
7. Cauldwell, R., and J. McLaughlin. 1984. Redistribution of Na,K-ATPase in the dystrophic rat retinal pigment epithelium. *J. Neurocytol.* 13: 895–910.
8. Chu, P., and G. Grunwald. 1990. Identification of an adhesion associated protein of the RPE. *Invest. Ophthalmol. Vis. Sci.* 31:847–855.
9. Citi, S., H. Sabanay, R. Jakes, B. Geiger, and J. Kendrick-Jones. 1988. Cingulin: a new peripheral component of tight junctions. *Nature (Lond.).* 333:272–276.
10. Dowling, J. E. 1960. Chemistry of visual adaptation in the rat. *Nature (Lond.).* 188:114–118.
11. Emanuel, J. R., S. Garetz, L. Stone, and R. Levenson. 1987. Differential expression of Na^+, K^+-ATPase alpha and beta subunit mRNAs in rat tissues and cell lines. *Proc. Natl. Acad. Sci. USA.* 84:9030–9034.
12. Feinberg, A. P., and B. Vogelsteing. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266–269.
13. Feramisco, J., J. Heifman, J. Smart, K. Burridge, and G. Thomas. 1982. Co-existance of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. *J. Biol. Chem.* 257:11024–11031.
14. Geering, K., D. I. Meyer, M. P. Paccolat, J. P. Kraehenbuhl, and B. C. Rossier. 1985. Membrane insertion of alpha and beta subunits Na,K-ATPase. *J. Biol. Chem.* 260:5154–5160.
15. Geering, K., I. Theulaz, G. Verrey, M. T. Hauptle, and B. C. Rossier. 1989. A role for beta subunit in the expression of functional Na,K-ATPase in Xenopus oocytes. *Am. J. Physiol.* 257:C851–858.
16. Ghosh, S., A. C. Freitag, P. Martin-Vasallo, and M. Coca-Prados. 1990. Cellular distribution and differential gene expression of the three alpha subunit isoforms of the Na,K-ATPase in the ocular ciliary epithelium. *J. Biol. Chem.* 265:2935–2940.
17. Herron, W., B. Riegel, and R. M. Myerso. 1969. Retinal dystrophy in the rat: a pigment epithelial disease. *Ophthalmology.* 8:595–604.
18. Jorgensen, P. L. 1982. Mechanism of the Na^+, K^+ pump. Protein structure and conformation of the pure (Na^+, K^+) ATPase. *Biochim. Biophys. Acta.* 694:27–68.
19. Katz, M. L., and W. G. Robison, Jr. 1985. Senescence and the retinal pigment epithelium: alterations in basal plasma membrane morphology. *Mech. Aging Dev.* 30:99–105.
20. Koob, R., M. Zimmermann, W. Schoner, and D. Drenckhahn. 1987. Colocalization and coprecipitation of ankyrin and Na^+,K^+-ATPase in kidney epithelial cells. *Eur. J. Cell Biol.* 45:230–237.
21. Kyte, J. 1976. Immunoferritin determination of the distribution of Na,K-ATPase over the plasma membranes of renal convulated tubules. *J. Cell Biol.* 68:287–303.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
23. Le Bivic, A., F. X. Real, and E. Rodriguez-Boulan. 1989. Vectorial targeting of apical and basolateral plasma membrane proteins in a human adenocarcinoma epithelial cell line. *Proc. Natl. Acad. Sci. USA.* 86:9313–9317.
24. Martin-Vassallo, P., W. Dockowski, J. Emanuel, and R. Levenson. 1989. Identification of a putative isoform of the Na,K-ATPase beta subunit. *J. Biol. Chem.* 264:4613–4618.
24a. Mayerson, P., M. Hall, V. Clark, and T. Abrams. 1985. An improved method for isolation and culture of rat retinal pigment epithelial cells. *Invest. Opthalmol. & Visual Sci.* 26:1599–1609.
25. McDonough, A., K. Geering, and R. Farley. 1990. The sodium pump needs its beta subunit. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:1598–1605.
26. McGrail, K., and K. Sweadner. 1986. Immunofluorescent localization of two different Na,K-ATPase in the rat retina and in identified dissociated retinal cells. *J. Neurosci.* 6:1272–1283.
27. McNeil, J., M. Ozawa, R. Kemler, and J. Nelson. 1990. Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell.* 62:309–316.
28. Miller, K. M., K. Simons, and D. L. Guyton. 1987. Television retinoscopy with a slit-aperture retinoscope and a highly sensitive camera. *Ophthalmology.* 2-P 22-7.
29. Deleted in proof.
30. Morro, J. S. 1989. The spectrin membrane skeleton: emerging concepts. *Curr. Opin. Cell Biol.* 1:23–29.
31. Morrow, J. S., C. D. Cianci, T. Ardito, A. S. Mann, and M. Kashgarian. 1989. Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin–Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–465.

32. Mullen, R. J., and M. M. La Vail. 1976. Inherited retinal dystrophy: primary defect in pigment epithelium determined with rat chimeras. *Science (Wash. DC).* 192:799–801.
33. Nelson, W. J., and P. J. Veshnock. 1986. Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin–Darby canine kidney epithelial cells. *J. Cell Biol.* 103:1751–1765.
34. Nelson, W. J., and P. J. Veshnock. 1987. Modulation of fodrin (membrane skeleton) stability by cell–cell contact in Madin–Darby canine kidney epithelial cells. *J. Cell Biol.* 104:1527–1537.
35. Nelson, W. J., and P. J. Veshnock. 1987. Ankyrin binding to Na^+, K^+, ATPase and implications for the organization of membrane domains in polarized cells. *Nature (Lond.).* 328:533–536.
36. Nelson, W. J., and R. W. Hammerton. 1989. A membrane–cytoskeletal complex containing Na,K-ATPase, ankyrin, and fodrin in Madin–Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108:893–902.
37. Nelson, W. J., E. M. Shore, A. Z. Wang, and R. W. Hammerton. 1990. Identification of a membrane–cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin–Darby canine kidney epithelial cells. *J. Cell Biol.* 110:349–357.
38. Noguchi, S., M. Noda, K. Kawakami, T. Ohta, K. Nagano, T. Hirose, S. Inayamas, M. Kawamura, and S. Numa. 1986. Primary structure of the beta subunit of Torpedo Californica. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 196:315–320.
39. Deleted in proof.
40. Orlowski, J., and J. B. Lingrel. 1988. Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic alpha isoform and beta subunit mRNAs. *J. Biol. Chem.* 263:10436–10442.
41. Philp, N., and M. Y. Yoon. 1990. The role of integrin in the attachment of RPE cells to basement membrane components. ARVO abs.
42. Philp, N. J., and V. T. Nachmias. 1985. Components of the cytoskeleton in the retinal pigmented epithelium of the chick. *J. Cell Biol.* 101: 358–362.
43. Renart, J., J. R. Reiser, and G. R. Stark. 1979. Transfer of proteins from gels of diazobenzyloxymethyl-paper and detection with antisera. *Proc. Natl. Acad. Sci. USA.* 76:3116–3120.
44. Rodriguez-Boulan, E. 1983. Polarized assembly of enveloped viruses from cultured epithelial cells. *Methods Enzymol.* 98:486–501.
45. Rodriguez-Boulan, E., and W. J. Nelson. 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science (Wash. DC).* 245:718–725.
46. Rodriguez-Boulan, E., and M. Pendergast. 1980. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell.* 20:45–54.
47. Schneider, B., E. Kraig, and D. Papermaster. 1988. Identification of isozymes of Na,K-ATPase in rat photoreceptors. *ASCB Abs.* 107(No.6, Pt.3):297a.
48. Shull, G. E., J. Greeb, and J. B. Lingrel. 1986. Molecular cloning of three distinct forms of the Na^+, K^+,-ATPase alpha subunit from rat brain. *Biochem. J.* 25:8125–8132.
49. Shull, M. M., and J. B. Lingrel. 1987. Multiple genes encode the human Na^+, K^+-ATPase catalytic subunit. *Proc. Natl. Acad. Sci. USA.* 84: 4039–4043.
50. Shyjan, A., C. Gottardi, and R. Levenson. 1989. The Na,K-ATPase beta-2 subunit is expressed in rat brain and copurifies with Na,K-ATPase activity. *J. Biol. Chem.* 265:5166–5169.
51. Steinberg, R. M., and S. S. Miller. 1979. Transport and membrane properties of the retinal pigment epithelium. *In* The Retinal Pigment Epithelium. K. M. Zinn and M. F. Marmor, editors. Harvard University Press, Cambridge, MA. 205–225.
52. Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.
53. Sweadner, K. 1989. Isozymes of the Na,K-ATPase. *Biochim. Biophys. Acta.* 988:185–220.
54. Deleted in proof.
55. Takeyasu, K., M. M. Tamkum, K. Renaud, and D. M. Fambrough. 1988. Ouabain sensitive (Na^+ + K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha subunit of an avian sodium pump. *J. Biol. Chem.* 263:4347–4354.
56. Tamkun, M. M., and D. M. Fambrough. 1986. The (Na^+ + K^+)-ATPase of chick sensory neurons. *J. Biol. Chem.* 261:1009–1019.
57. Tate, S., L. Ross, and A. Meister. 1973. The gamma glutamyl cycle in the choroid plexus: its possible action in amino acid transport. *Proc. Natl. Acad. Sci. USA.* 70:1447–1449.
58. Tokuyaou, K. T. 1973. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551–565.
59. Towbin, H. T., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
60. van Deurs, B., K. Sandvig, O. W. Petersen, S. Olsnes, K. Simons, and G. Griffiths. 1988. Estimation of the amount of internalized ricin that reaches the *trans*-Golgi network. *J. Cell Biol.* 106:253–267.
61. Wandinger-Ness, A., and K. Simons. 1990. Polarized sorting in epithelia. *Cell.* 62:207–210.
62. Yang-Feng, T., J. Schneider, V. Lindgren, V. Shull, E. Benz, J. Lingrel, and U. Francke. 1988. Chromosomal localization of human Na,K-ATPase alpha and beta subunit. *Genomics.* 2:128–138.
63. Young, R., and J. Lingrel. 1987. Tissue distribution of mRNAs encoding the alpha isoforms and beta subunit of rat Na^+, K^+-ATPase. *Biochem. Biophys. Res. Commun.* 145:52–58.
64. Young, R. W., and D. Bok. 1969. Participation of the retinal pigment epithelium in the rod outer segment renewal process. *J. Cell Biol.* 42:392–403.
65. Zinn, K. M., and M. F. Marmor. 1979. The Retinal Pigment Epithelium. Harvard University Press, Cambridge.